Does Successful Allopregnancy Mimic Transplantation Tolerance?

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The dendritic-cell associated molecule CD200 is up-regulated in rodent transplantation models where successful inhibition of rejection is accomplished. The mechanism by which CD200 achieves this effect involves signaling a receptor, $CD200^r$, on macrophages and/or $\gamma\delta TCR^+$ cells, both of which have been implicated in adoptive transfer of tolerance. In addition to inhibition of rejection, increased expression of CD200 is associated with altered polarization in cytokine production, with increased expression of IL-4, IL-10 and TGFβ, and decreased IL-2, IFNγ and TNF-α. Inhibition of rejection can thus be adoptively transferred by IL-10/TGFβ and a CD200 immunoadhesin and in turn can be inhibited by neutralizing these cytokines or by functional blockade of CD200 expression.

Successful pregnancy in allopregnant mice can also be viewed as dependent upon control of graft rejection. Proinflammatory Th1 cytokines (TNF-α + IFN-γ + IL-1) can cause spontaneous abortion in mice by a mechanism which involves a novel prothrombinase, fgl2, which promotes fibrin deposition. However, we found that spontaneous abortion rates in abortion-prone CBA x DBA/2 matings and in low abortion rate CBA x BALB/c matings were lower than the frequency of implantation sites showing $fibrin^{hi}$ + $fgl2\ mRNA^{hi}$. CD200 expression was present in the same sites as fgl2 mRNA, and neutralization of this CD200 expression by anti-CD200 antibody raised the abortion rate to predicted levels. Conversely, a CD200 immunoadhesin dramatically reduced the abortion rate. We hypothesize that in addition to its role in organ and tissue allograft rejection, CD200 expression is involved in the prevention of spontaneous abortion triggered by cytokine up-regulation of fgl2 at the feto-maternal interface.

Introduction

Generally the implanted semi-allogeneic embryo can be viewed as a successful allograft which is not spontaneously rejected.[1,2] Successful survival, in both pregnancy[3-5] and organ transplantation,[6-8] has been associated with a bias toward (local) type-2 cytokine production. In contrast, when graft (or fetal) rejection occurs, Th1-type proinflammatory cytokines (e.g., IL-1, IFN-γ, TNF-α) have been shown to play a predominant role.[1,6,7,9,10] Recent studies on the mechanism of pregnancy termination have also shown a role for cytokine activation of a novel prothrombinase, fgl2, which generates thrombin. The latter in turn leads to clotting and activation of polymorphonuclear leukocytes (PMNL), which together terminate the blood supply to the developing placenta.[11] In support of this hypothesis, the increased rate of spontaneous abortions occurring in CBA × DBA/2 matings, which depend upon IL-1, TNF-α and IFN-γ, is abrogated by the anti-fgl2 neutralizing antibody, heparin and/or hirudin, and also by the monoclonal anti-PMNL antibody.[11]

Recent studies in the mouse by Ito et al[12] have implicated a Vα14 NKT cell population, which is stimulated by a CD1d restricted ligand, α-galactosylceramide (αGalCer), to produce type-1 cytokines (IFNγ, TNF-α) in abortion. A homologous population of $CD3^+CD161^+V\alpha24^+$ NKT cells, recognizing the same ligand, has been identified in humans by Tsuda et al.[13] As further documentation of the importance of this cell population in regulation

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of immunity during pregnancy, Ito et al[12] reported that animals carrying homologous deletions for Vα14 NKT cells (or indeed for the genes encoding IFNγ, TNF-α or perforin) showed a loss of αGal-Cer-triggered abortions. Interestingly, in the CBA × DBA/2 model, NK and Vγ1.1δ6.3NKT cells produce IFNγ and TNF-α in response to as yet unidentified ligands on fetal trophoblast cells.[14-17] In the CBA × BALB/c matings, which have a low abortion rate, pre-implantation activation of maternal $CD8^+$ ($V\gamma^{1+}$) "suppressor" T cells inhibits NK and NKγδT cell activation.[1,2,18] In contrast, in the CBA × DBA/2 mating combination, there is deficient activation of these $CD8^+$ suppressor cells, and a population of IL-10 and TGF-β-producing γδ suppressor cells developing in pregnancy decidua 4-5 days after implantation (grafting) has been implicated in rescuing potentially doomed implantations from abortion.[15,16] We had suggested that a similar population of γδ cells was implicated in the regulation of organ transplant rejection in rodent model systems[18,19] where Vαβ $CD4^+$ and $CD8^+$ cells play the key effector role. Regardless of whether a $CD8^+$ or $CD8^-$ suppressor T cell is responsible for regulation of the abortion-inducing cells, these data suggest that this regulation of activation of Th1 cytokine-producing cells plays a paramount role in the final outcome of the allograft.

Productive activation of αβT cells occurs after concomitant engagement of TCR αβ with antigen presented on antigen presenting cell (APC) in association with major histocompatibility complex (MHC) molecules and the delivery of costimulatory signals resulting from the interaction of several ligand:coreceptor complexes.[20,21] Major positive costimulatory interactions include the following: CD40L with CD40 and CD28 with CD80/CD86; CTLA4 interactions with CD80/CD86 may deliver a negative signal.[22-24] TCR $\gamma\delta^+$ cells may recognize antigen in absence of MHC, or in association with MHC (usually class Ib), and the need for positive costimulation signals is less certain. While positive costimulatory signals are clearly important in T cell triggering, blocking this costimulation alone, and/or facilitating signaling via CTLA4, has not reproducibly induced αβT cell tolerance. We have suggested that this reflects the role for other molecules in active immunoregulation and have identified one such molecule, OX2 (hereafter referred to by its new CD designation, CD200).[25] Dendritic cells (DC) expressing CD200 triggered an immunoregulatory function leading to increased allograft survival.[26] Moreover, these regulatory cells were physically distinguishable from those DC with optimal allostimulatory capacity.[27] The regulation triggered by CD200 is dependent upon engagement of a receptor (hereafter referred to as $CD200^r$) on other populations of cells (the proximal suppressor cell(s)), one of which has been identified in an organ transplant system as an $F4/80^+$ macrophage, and another as an activated γδ T cell.[28]

In the studies detailed below, we show evidence, using anti-CD200 monoclonal antibody and a soluble form of CD200 in which the extracellular domains of the molecule are linked to an Ig Fc region (CD200:Fc),[29] that this same CD200:$CD200^r$ interaction is fundamentally important to achieving successful allopregnancy.

CD200:

Ig supergene family member, expressed on follicular dendritic cells, activated lymphocytes, endothelium, neurons, which triggers an immunosuppressive signal in CD200R-bearing cells.

ALLOPREGNANCY:

Offspring of mating between two genetically disparate members of the same species.

Method

All of the techniques used, including mixed leukocyte cultures, cytokine analysis and allografting, are detailed in previous publications.[8,16,17] The anti-CD200 mAb (3B6) was obtained from BioCan (Mississauga, Ontario).[26] 100 μg/mouse was used for each injection. A polyclonal, affinity-purified, rabbit antibody to fgl2 was described elsewhere, and used ip at a dose of 22 μg/mouse.[11] CD200Fc immunoadhesin[29] was given ip (35 μg/mouse).

Results

In Situ Expression of CD200 mRNA Following Renal Transplantation or Allopregnancy

CD200 expression has been reported at the feto-maternal interface using immunohistochemistry in rats.[9] To determine whether CD200 was expressed in the uterus of allopregnant mice, we carried out in situ hybridization for CD200 mRNA in CBA × DBA/2 and CBA × BALB/c matings. Adjacent sections of the tissue samples were also used to stain for fgl2 mRNA (fgl2 is a prothrombinase molecule up-regulated by certain Th1 cytokines implicated in triggering pregnancy loss). For comparison, we also examined CD200 expression in liver sections from C3H mice receiving C57BL/6 renal allografts follow-

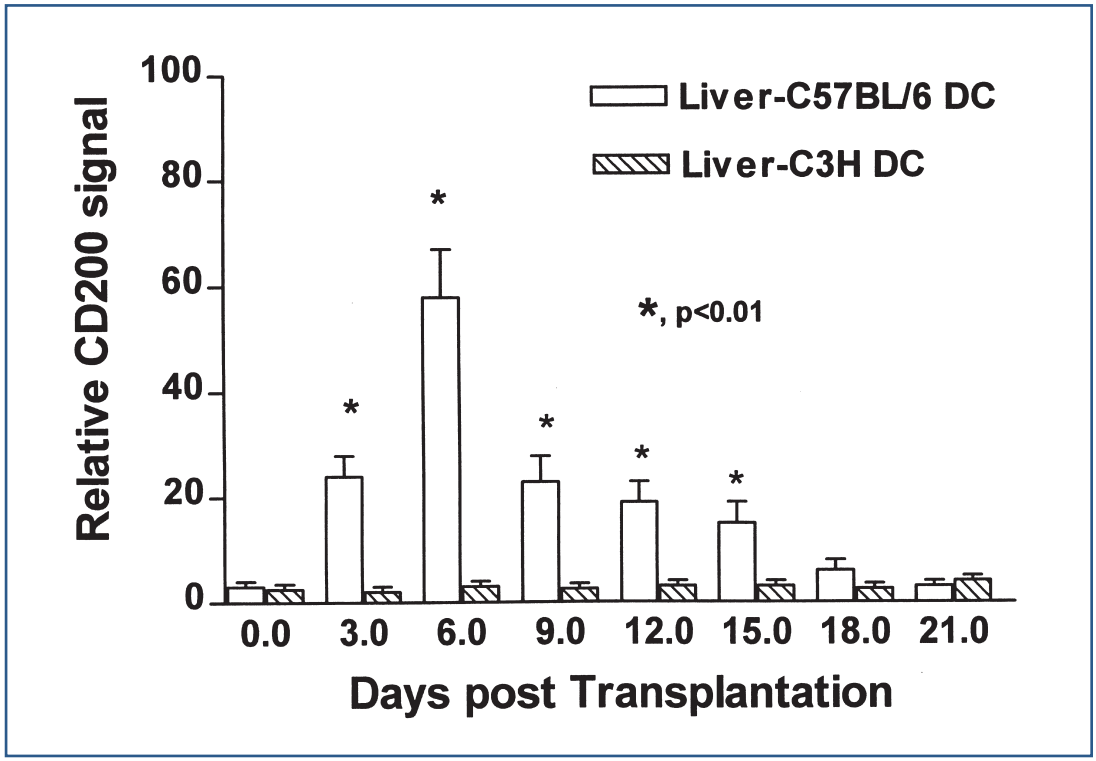

Figure 1. Kinetics of expression of mRNA for CD200 in liver tissue of recipient mice following pv infusion of syngeneic or allogeneic dendritic cells (DC) and renal transplantation as determined by quantitative PCR. All data (mean±SD from three mice/group) were compared using mRNA from tissue harvested 6 days after pv immunization with C57BL/6 DC, after first normalization to a standard signal for GAPDH.*p<0.01 compared with syngeneic DC.

ing donor-specific portal vein pre-immunization, a treatment which promotes tolerance and which is critically dependent upon up-regulation of expression of CD200 on hepatic APC.[7] Typical patterns for uterine staining (pregnant mice) are reported elsewhere,[30] along with cumulative data for CD200 and fgl2 expression in the uteri of pregnant control mice and mice treated with TNF-α + IFNγ to increase abortion rates.[30]

In these studies we found that CD200 mRNA expression was up-regulated following pv immunization and renal transplantation and in allopregnant mice. In pregnant mice we found a negative correlation between expression of the molecules fgl2 and CD200, which did not reach statistical significance.[30] Following cytokine treatment of pregnant animals, and prior to the onset of abortions, the proportion of $fgl2^{hi}$ implantations increased, although this also did not achieve significance due to small numbers. However, with cytokine treatment, the proportion of $CD200^{hi}$ implants decreased dramatically. These data support the hypothesis that in pregnancy, fgl2 and CD200 expression are reciprocally regulated by cytokines, that their levels affect pregnancy outcome and that a major determinant of success or failure of $fgl2^{hi}$ at-risk implantations was the presence or absence of CD200. Most interestingly, we also reported that continued expression of CD200, as occurs in pregnancy, was essential for successful survival of allografts following pv pretransplant immunization and for the concomitant changes in cytokine production seen in those animals[31] (see also Fig. 1).

Effect of Anti-CD200 mAb on Renal Transplant Survival and Pregnancy Outcome

Fifty to 70% of implantations in control and cytokine-boosted CBA × DBA/2 pregnancies show the $fgl2^{hi}$ phenotype.[30] An injection of anti-Vγ1.1 on day 8.5 of pregnancy, 1 day before abortions become evident, inactivates most of the trophoblast-recognizing γδ subset producing IL-10 and TGFβ and boosts abortion rates to approximately 48%.[15] We hypothesized that the suppressor γδT cells inactivated by this treatment might be dependent upon CD200 expression for their functional activ-

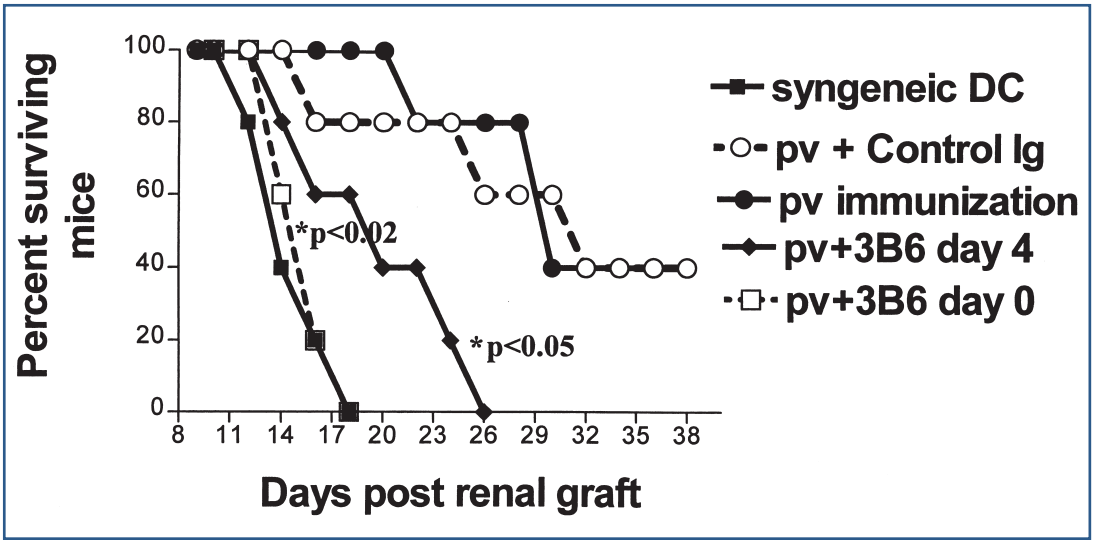

Fig 2. Anti-CD200 mAb (3B6) reverses increased renal allograft survival following pv immunization. Five mice per group received renal allografts after pretransplant pv immunization with syngeneic (control) or allogeneic dendritic cells (DC). Some mice also received infusions of control Ig or 3B6 (100 μg/injection) beginning at day 0 or 4 after transplantation. * shows p values relative to mice not receiving anti-CD200 (3B6) antibody (●,○).

ity. We have reported that following allotransplantation, the kinetics of expression of CD200 (see Fig. 1 for mRNA expression in liver of CD200 following pv immunization and renal transplantation) follows closely the development of immunoregulatory γδT cells.[31] Functional blockade of CD200 expression (by anti-CD200 treatment) reverses increased grafts survival (see Fig. 2) and prevents adoptive transfer of tolerance by γδT cells. To test whether CD200 expression in pregnancy might similarly be activating anti-abortion mechanisms which rescued fgl2^{hi} implant sites from proceeding to embryo death where both CD200 and fgl-2 were expressed, we injected control CBA × DBA/2- and CBA × BALB/c-mated mice with the same anti-CD200 monoclonal antibody that blocks induction of transplantation tolerance. Fig. 3 shows that injection on or after day 8.5 increased the spontaneous abortion rate to that expected if all fgl2^{hi} sites in CBA × DBA/2 proceeded to resorb. The increase in abortion rate was not due to a toxic effect of anti-CD200 on the embryo because co-administration of anti-fgl2 to neutralize prothrombinase activity abrogated the boost in abortion rates[30] (data not shown). Injection of anti-CD200 into CBA × BALB/c-mated mice also increased the abortion rate to 22%, consistent with the 21% fgl2 mRNA^{hi} implantation site frequency.[30]

TRANSPLANTATION TOLERANCE:

Specific inhibition (loss) of immunity to foreign graft, without reduction in general immune responsiveness.

Infusion of CD200 immunoadhesin modulates renal allograft rejection and spontaneous abortion

As further proof of principle that CD200 expression is functionally important for increased allograft survival, we used an immunoadhesin (CD200:Fc), in which the extracellular domains of CD200 were linked to a murine IgG2aFc region, to investigate modulation of allograft rejection and pregnancy. Previous data has already indicated that this molecule has potent immunoregulatory properties in vivo, including the ability to decrease allograft rejection.[29] Data in Fig. 4 represent a cumulative comparison of the effect of infusion of CD200:Fc on renal allograft survival or rate of abortions in CBA × DBA/2-mated mice. Once again there was a clear parallel between the functional activity of CD200:Fc measured in these two assays.

Discussion

The notion that the rejection of organ allografts would be mimicked immunologically by immune recognition of the fetus in allopregnant mothers has

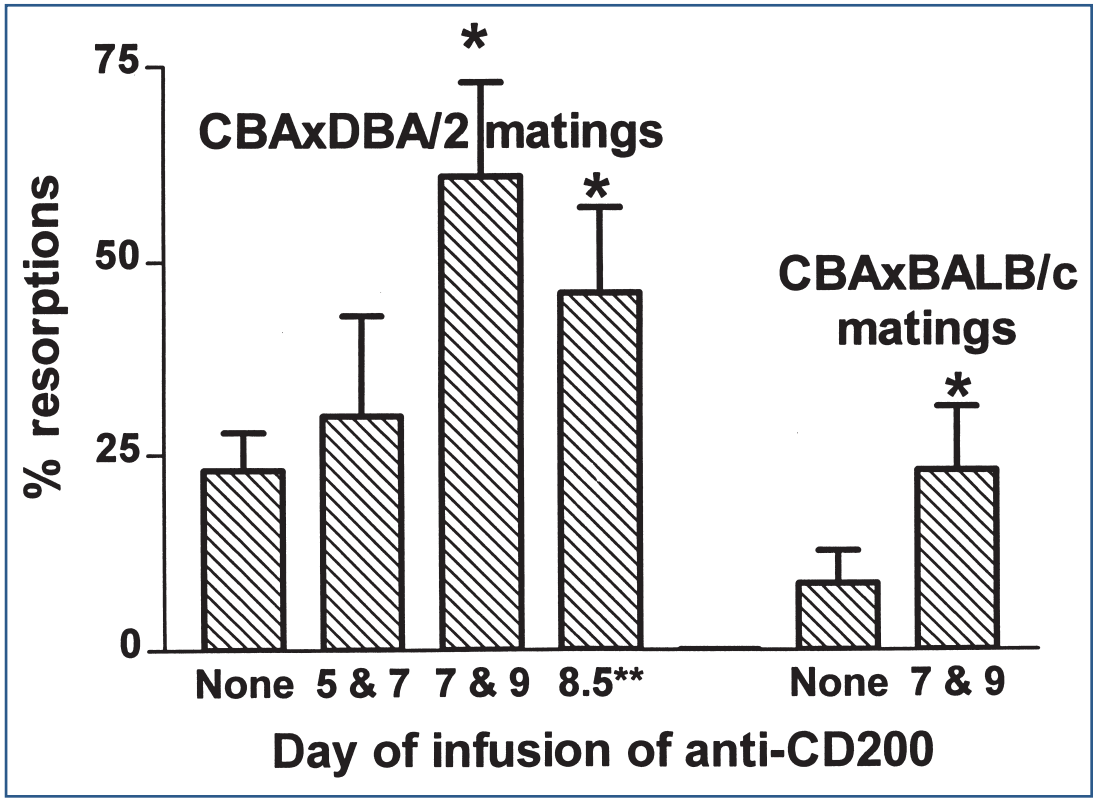

Figure 3. Effect of anti-CD200 monoclonal antibody 3B6 on spontaneous resorption (abortion) rates. Error bars show 1 standard deviation. A minimum of 20 implants was scored for each group. * significant increase in abortion rate, $p < 0.02$. **single injection of antibody only (day 8.5). Modified from data published in Clark et al[30]; published with permission from *Molecular Human Reproduction*.

CYTOKINES:

Molecules used by cells within the immune system to activate/regulate specific and non-antigen-specific immune responses.

been with us for decades. There has been intense interest in the role of altered cells and soluble factors (e.g., cytokines) in the phenomena seen in both situations. As an example, with decreased graft rejection (and successful pregnancy) there are numerous reports of the presence of unique γδT cells with suppressor phenotypes, and altered cytokine patterns, with elevated levels of type-2 cytokines, in particular IL-10 and TGFβ.[4,13-16] In contrast, allograft rejection in rodents and man has been associated with elevated type-1 cytokines, and previous studies have also shown that both TNF-α and IFNγ must be present for spontaneous abortions to be induced.[4] Since the fgl2 gene is activated by IFNγ but not by TNF-α, we have hypothesized that the obligatory role for the latter cytokine either involves activation of PMNL essential for abortions to be completed[4] or down-regulates CD200 expression. These issues are currently under investigation.

It is worthy of note that in our previous report, and in the studies described above, expression of fgl2, a thrombosis-inducing molecule, was up-regulated on the trophoblast in response to cytokines. Cytokine-treated IRF1$^{-/-}$ females mated to +/+ males do not abort, as there is no up-regulation of fgl2 in the maternal decidual tissues and the best explanation for lack of abortions is that fgl2^{+} trophoblast and maternal decidua must meet. In the regions where the two tissues meet, a zone of spontaneous cleavage, enough enzymatic activity presumably occurs to cause necrosis. We have also documented a basal level of fgl2 expression in trophoblast tissue.[30] Given the evidence that excessive anticoagulation with heparin or hirudin leads to retroplacental hemorrhage fatal to the embryo and sometimes mother,[5]

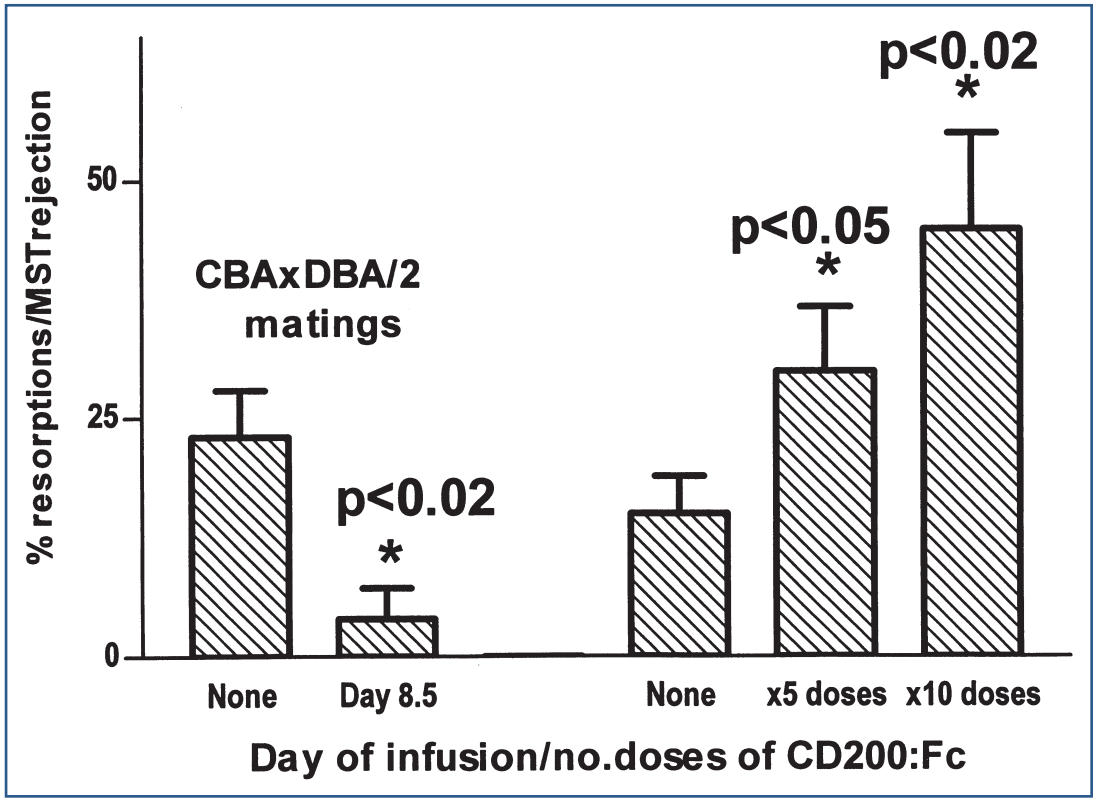

Figure. 4. Effect of CD200 immunoadhesin (CD200:Fc) on spontaneous resorption (abortion) rates or renal allograft rejection. Error bars show 1 standard deviation. A minimum of 45 implants was scored for each group of pregnant mice (mice received a single dose of CD200:Fc at day 6.5; 10 mice/group were used for renal allografts, and mice received multiple iv injections of CD200:Fc, beginning on the day of transplant and at 2-day intervals thereafter. * significant decrease in abortion rate, or increased graft survival, relative to controls.

we suggest that this low (basal) level of expression of the molecule fgl2 may reflect a normal homeostatic role for fgl2 in preventing spontaneous bleeding. However, cytokine-mediated (by TNF-α and IFNγ) up-regulation of fgl2 is associated with increased rates of abortion.[19] These effects can in turn be counteracted by the combination of both TGF-β and IL-10, both perhaps produced by trophoblast cells, which are known to inhibit cell-mediated vascular injury and clotting.[13,14-17] There is little data to date examining the role of fgl2 prothrombinase in transplant rejection, though preliminary data suggest an up-regulation of expression of fgl2 expression in rejecting allografts.[32] Interestingly, xenograft rejection, a process in which acute and subacute vascular changes are believed crucial, differs both in quality and in tempo in an fgl2 knockout mouse (Levy et al, personal communication). It thus becomes extremely interesting to know whether fgl2 has a more general role in immunomodulation in both allo and fetal grafts.

Our present data shed further light on the mechanisms by which some of these changes occur in both the allopregnant mouse and in allotransplants, by providing evidence of a crucial role for altered expression of another molecule CD200 in regulating both embryo execution triggered by cytokine up-regulation of fgl2 prothrombinase and the modulation of renal allograft rejection. We have proposed that CD200 acts in transplantation as a costimulatory signal that deviates cytokine production away from Th1 (e.g., IL-2, IFN-γ) and toward Th2/3 (e.g., IL-4, IL-10, TGF-α) production.[25-29] Associated with this is the expansion of a γδT cell subset that mediates tolerance via suppression.[18,19] In support of such a hypothesis, we have shown

that increased expression of CD200 is associated with decreased rejection and altered cytokine production, that the kinetics of expression of CD200 parallels altered cytokine production and γδT cell expansion and that these effects are diminished by infusion of anti-CD200 mAbs and enhanced by infusion of the immunoadhesin CD200:Fc (see also Figs. 1, 2 and 4). Note that most of our transplant data comes from models of renal allotransplantation. There is some evidence suggesting that renal allografts in rodent models may allow for easier "tolerization" than other solid organ (e.g., cardiac) allografts. However, we have also published extensively on the similarity of the data on CD200 seen in models of skin allograft rejection (a much more immunogenic model), as well as in small intestinal allografts in rat and mouse cardiac allografts (Gorczynski, unpublished data), confirming the general relevance of our data to other models.[18,19,33] We have also documented that a similar correlation exists between CD200 expression and fetal loss in allopregnant mice, even when abortion rates are increased following infusion of cytokines (where our data suggests CD200 continues to act to counter the effects of fgl2[30]).

Since antigen-specific responses by the mother to paternal antigens are usually protective against pregnancy loss, and the driving force behind miscarriages that depend on fgl2 up-regulation is lipopolysaccharide, we would expect that anti-CD200 would increase losses in a syngeneic system and, conversely, that CD200:Fc would reduce losses. To date, preliminary studies suggest that indeed anti-CD200 increases fetal loss, interestingly in both the homozygous (C3H × C3H) and (CBA × CBA) mating combinations. Since C3H/ HeJ mice lack TollR4 expression, a key receptor for the lipid A of LPS, and indeed do not show increased fetal loss in response to LPS injection, there may be more than one pathway to fgl2-dependent fetal loss, including perhaps other Toll pathways which might substitute for TLR4 and lead to stimulation of Th1 cytokines which promote expression of fgl2. The potential role for an immune response to minor histocompatibility antigen differences, which may be implicated in these scenarios and possibly even in the allopregnant mice, remains unclear. In CBA × DBA/2 matings, minor antigen differences are thought to predispose to fetal loss, but they seem to act primarily via NK, γδTCR$^+$ cells and NKγδT cells. Experiments to examine in more detail the pathways mediating fetal loss in anti-CD200-treated syngeneic matings are in progress.

It is now known that CD200 functions following interaction with its receptor (CD200^r) on target cells. At least two groups, ourselves[28] and Wright et al,[34] have documented the existence of CD200^r on macrophages, and we showed optimal inhibition of graft rejection in vivo occurred with infusion of both CD200:Fc and CD200^{r+} cells.[28] Unlike the Barclay group, we have found that a large percentage (>80%) of ConA-activated γδT cells also express a CD200^r as defined by FACS with FITC-CD200:Fc. We have recently confirmed this independently using our mAbs to CD200^r, and following cDNA sequencing of the CD200^r expressed in γδTCR$^+$ hybridomas (Yu et al: manuscript in preparation). We have not yet studied the functional activity of CD200^r cells (whether macrophages or γδT cells) in allopregnant mice. However, extrapolating from the data shown above, we suggest that increased expression of CD200^r will correlate with successful allopregnancy, and that triggering intracellular signaling by cross-linking CD200^r on cells by mAb (presumably in the same fashion as native cell- bound CD200 does when it interacts with CD200^r) will protect mice from cytokine-induced spontaneous abortion (mediated by elevated fgl2 expression). Similar results are anticipated in our renal transplant model using anti-CD200^r.

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